- Replace the paragraph at page 7, line 22 to page 8, line 3 with the following:

--Figure 3 demonstrates rescue of the expression of a derivative of the CBP-Cre fusion protein by introduction of the *ileY* gene. Cultures of BL21gold DE3 cells containing T7-driven vectors for a CBP/Cre-recombinase fusion gene with either three rare leucine (CBP-3xL-Cre, codon CUA) or isoleucine codons (CBP-3xL-Cre, codon AUA) at the 5' end of the gene, and the indicated pACYC-based tRNA expression vectors were induced at mid-log phase for 1h with 1mM IPTG. Fifteen µl of whole cell lysate were loaded on a 4-20% PAGE gel and stained with Coomassie blue. The position of the induced fusion protein is indicated by an arrow. A sample of CBP-Cre without any extra codons was added as a reference. The lanes labeled with RG contain an expression vector for the tRNA genes *argU* and *glyU*. The lanes labeled RIL contain an expression vector for the *argU, ileY* and *leuW* tRNA genes.--

- Replace the paragraph on page 8, lines 4-16 with the following:

--Figure 4 demonstrates the functionality of the *proL* gene in an RILP array. Cultures of BL21goldDE3 strains containing pACYC-based vectors expressing copies of the indicated *E.coli* tRNA genes and the T7-driven expression vectors for human cardiac troponin T (*argU*-dependent) and the Cre-recombinase/CBP fusion genes CBP-exi-Cre (*ileY*-dependent) and CBP-3xP-Cre (*proL*-dependent) were induced for 2h with 1mM IPTG. Fifteen µl of whole cell lysate were loaded on 4-20% PAGE-gels and stained with Coomassie blue. HcTnT is an *argU*-dependent T7-driven recombinant human cardiac troponin T expression construct (Hu et al.). CBP-3xi-Cre and CBP-3xP-Cre are T7-driven CBP-Cre-recombinase fusion genes containing either three AUA(3xi) codons or three CCC(3xP) codons near the N-terminus of the fusion protein. Cells with the RILP array (containing the *proL* tRNA gene), but not the RIL array, allow efficient expression of the recombinant CBP-3xP-Cre gene. The arrows indicate the position of the recombinant gene products. RIL: *argU, ileY* and *leuW* tRNA genes. RILP: *argU, ileY, leuW*, and *proL* tRNA genes.--

- Replace the paragraph on page 8, lines 17 to 25 with the following:

--Figure 5 reveals that the RIL and RILP arrays do not significantly affect the level of expression of well-expressed proteins. The indicated host cells were transformed by T7-driven

expression vectors for JNK (human c-jun N-terminal kinase), λ-phosphatase or calmodulin. Cultures were induced at mid-log growth for 2h with 1mM IPTG. Fifteen µl of induced cultures were denatured by boiling in SDS loading buffer, separated on a 4-20% PAGE gel and stained with Coomassie blue. The positions of the induced heterologous proteins are indicated by arrows. "-": BL21gold DE3. RIL: BL21gold DE3 with the *argU, ileY* and *leuW* tRNA gene array. RILP: BL21gold DE3 with the *argU, ileY, leuW* and *proL* tRNA gene array.--

- Replace the paragraph on page 8, line 26 to page 9, line 10 with the following:

--Figure 6 shows that high level expression of *Pfu*-polymerase depends on the presence of extra copies of both the *argU* and *ileY* genes. Cultures of BL21gold DE3 strains containing pACYC-based vectors expressing copies of the indicated *E.coli* tRNA genes and the T7-driven expression vectors for *Pfu*-polymerase, human cardiac troponin T and CBP/Cre-recombinase were induced for 2h with 1mM IPTG. Fifteen µl of whole cell lysate were loaded on 4-20% PAGE-gels and stained with Coomassie blue. High level of expression of human cardiac troponin T (hcTnT) in *E. coli (*expressed from a pET21b construct) is dependent on removal of two tandem AGA/AGG codons or rescue by extra copies of the *argU* tRNA gene. CBP-3xi-Cre is a CBP-tagged Cre-recombinase construct containing 3 extra AUA codons (coding for isoleucine) at the 5' -end of the recombinant gene. High level expression of this protein in *E. coli* is dependent on the presence of extra copies of *E. coli IleY* t-RNA gene (see Fig. 3). RG: pACYC with *argU* and *glyU* tRNA genes. RI: *argU* and *ileY* tRNA genes. IL: *ileY* and *leuW* tRNA genes. RIL: *argU, ileY*, and *leuW* tRNA genes.—

- Replace the paragraph on page 9, lines 11 to 19 with the following:

--Figure 7 demonstrates that failure of the RG array to support efficient *Pfu* DNA-polymerase expression is not due to a negative effect of the *glyU* gene. The experiment displayed in Fig. 7 was repeated using an *argU/leuW* array (RL) instead of the *argU/glyU* array (RG), yielding the same result. Cultures of BL21goldDE3 strains containing pACYC-based vectors expressing copies of the indicated *E. coli* tRNA genes and the T7-driven expression vector for *Pfu* polymerase were induced for 2h with 1 mM IPTG. Fifteen µL of whole cell lysate were loaded on 4-20% PAGE gels and stained with Coomassie stain. RL: pACYC with *argU*

and *leuW* tRNA genes. RI: *argU* and *ileY* tRNA genes. IL: *ileY* and *leuW* tRNA genes. RIL: *argU*, *ileY*, and *leuW* tRNA genes.--

- Replace the paragraph on page 9, lines 20 to 29 with the following:

--Figure 8 indicates that Pfu DNA polymerase I expression correlates with functional *argU* and *ileY* expression. *Pfu* DNA polymerase I was expressed in BL21goldDE3 strains either containing no tRNA expression vector (-), pACYC-RIL, or two different isolates of the RILP expression vector. Both RILP isolates displayed functional *argU* expression at the same level as observed with pACYC-RIL vector. However, RILP9 displays no functional *ileY* expression, and in RILP16 functional *ileY* expression is diminished when compared to RIL. Functional *ileY* expression was evaluated by rescue of the *ileY*-dependent production of CBP-3xi-Cre. Fifteen µl of the indicated cultures induced at mid-log growth for 2h with 1 mM IPTG were loaded on a 4-20% PAGE gel and the separated proteins were visualized by Coomassie blue staining.--

- Replace the paragraph at page 10, lines 1 to 7 with the following:

--Figure 9 demonstrates that the expression of *Pfu*-polymerase II subunits I and II are dependent on functional co-expression of the *argU* and *ileY* genes. *Pfu* DNA polymerase I and the two sub-units of *Pfu*-DNA polymerase II were expressed in the indicated host strains. Fifteen µl of the cultures induced at mid-log growth for 2h with 1 mM IPTG were loaded on a 4-20% PAGE gel, and the separated proteins were visualized by Coomassie blue staining. "-": BL21gold DE3. RG: *argU* and *glyU* tRNA genes. RI: *argU* and *ileY* tRNA genes. IL: *ileY* and *leuW* tRNA genes. RIL: *argU, ileY,* and *leuW* tRNA genes.--

- Replace the paragraph at page 10, line 23 to page 11, line 6 with the following:

--This invention is directed toward the problem of rare codon usage in a host cell, which limits expression of a desired recombinant protein. "Codon usage" refers to the frequency with which a given codon appears in the coding regions of a gene. The codon usage of a host cell refers to the average codon usage for known genes which are endogenous to the host cell. A codon or its usage is "rare" if its frequency of use in the host cell is such that depletion of the corresponding tRNA species occurs during expression (particularly high level expression (see below)) of a heterologous protein of interest. A codon which is "rare" in a given host cell may

be one which is normally not used by the host cell at all or which is used by the host cell in less than 1% and even less than 0.5% of the host cell genes, or may be one which becomes limiting for the level of expression of a protein of interest. --

- Replace the paragraph at page 14, line 20 to page 15, line 8 with the following:

--Specialized arrays of tRNA genes biased towards the codon usage of specific organisms are utilized according to the invention. Frequently used codons (arbitrarily designated as codons with a frequency of more than 1.5%) in heterologous genes constitute potential limitations to heterologous protein expression in *E. coli*. Using tables prepared for other host cells, arrays of tRNA genes can be designed to meet the needs of a high level expression system for any organism. For example, one possible array for expression of human proteins in *E. coli* would include the cognate tRNA genes for codons AGG and AGA (Arg); CCC, CCU, and CCA (Pro); GGA and GGG (Gly); and UCC (Ser). Another example of an array that could be selected is the combination of arginine and proline codons (AGG and AGA (Arg); CCC (Pro)), which could be provided, for example, by the *E. coli* tRNA genes *argU* and *proL*. Yet another example of an array that could be selected is the combination of arginine, isoleucine, and leucine codons (AGG and AGA (Arg); AUA (Ile); and CUA (Leu)), provided, for example, by the *E. coli* tRNA genes *argU*, *ileY*, and *leuW*. By designing arrays of tRNA genes in this way, it is possible to tailor an expression host to meet the needs of any desired recombinant protein with a particular codon bias.--

- Replace the paragraph at page 15, line 24 to page 16, line 4 with the following:

--In a preferred embodiment of an array according to the invention, which targets the codons AGA or AGG (Arg)), CUA (Leu), AUA (Ile) and CCC (Pro) in *E. coli*, the choice of these codons is based on their infrequent use in *E. coli*, and the availability of the cognate tRNA genes. To prepare this embodiment, an array of four tRNA genes which encode tRNAs rarely expressed in *E. coli*, *argU, ileY, leuW and proL* (recognizing the codons AGA/AGG, AUA, CUA and CCC, respectively) was introduced into a low copy number plasmid, pACYC-LIC (see Example 1). These four genes, which are rare in *E. coli*, were isolated from *E. coli* K12. Introduction of this plasmid into suitable protein expression hosts such as *E. coli* BL21DE3 allows high level of expression of proteins normally restricted by the presence of rare codons.

- Replace the paragraph at page 27, line 19 to page 28, line 3 with the following:

--Since genes affected by AUA (ile), CGA (leu) or CCC (pro) were not available, tester constructs were generated based on the observation (Rosenberg, 1993, *supra*) that rare codons affect translation in *E.coli* most when present in a consecutive arrangement at the N-terminus of the protein. Three consecutive leucine (CUA), isoleucine (AUA), or proline (CCC) codons were introduced at the 5' end of the recombinant CBP/Cre fusion gene. Upon IPTG induction, unmodified CBP/Cre fusion protein was expressed at approximately 30% of the total protein, most, if not all of it being soluble. As can be seen in Figure 3, introduction of the rare leucine codon CUA did not affect expression of the recombinant gene. The functional expression of the *leuW* gene could therefore not be assessed. In contrast, introduction of the isoleucine codon strongly reduced expression of the recombinant protein. Expression could be rescued by expression of sets or arrays containing the *ileY* gene, but not by the RG *(argU and* glyU) set, which lacks the *ileY* gene. Thus, the *ileY* gene in the RIL array is functional and specifically rescues expression of genes affected by the rare isoleucine codon AUA.--

B12

Paragraph at page 28, lines 4 to 19:

--The same strategy was applied to test for functional expression of the *proL* gene in the RILP array. As can be seen in Figure 4, the presence of the RILP array, but not the RIL array, rescued expression of a tester construct containing the cognate CCC codon. Therefore, the *proL* gene in the RILP array is functional. However, although the *argU* gene in the RILP array performs at the same level as observed in the RIL array, functional expression of the *ileY* gene in the RILP array was diminished when compared to the RIL array. Five independent isolates of the pACYC-RILP construct were tested and the same effect was observed in all constructs, albeit to a different extent (ranging from undetectable expression to detectable, but diminished). Sequencing the RILP-tRNA gene arrays revealed that in isolates failing to show *ileY* activity, the *ileY* gene contains a point mutation (A --> T at nt 50 of the mature tRNA) that prevents proper folding of the tRNA and thus is likely to inactivate it. However, in the RILP isolates displaying diminished activity, the sequences of the *argU*, *ileY*, and *leuW* tRNA genes were indistinguishable from the RIL array. This observation suggests a potential incompatibility of simultaneous ectopic expression of the *proL* and *ileY* tRNA genes, possibly due to either

B13

interference of the *proL* gene with *ileY* transcription and/or processing or due to an attenuation of the host cells.--

- Replace the paragraph at page 28, line 22 to page 29, line 7 with the following:

--In order to test for potential deleterious effects of the RIL and RILP arrays, the expression of four well-expressing T7-driven recombinant genes were compared in BL21goldDE3 cells and their pACYC-RIL and pACYC-RILP containing derivatives. As shown in Fig. 5, no differences were observed between BL21goldDE3 cells and their tRNA-supplemented derivatives in the induced expression level of λ-phosphatase or JNK. However, a loss of expression was observed for calmodulin. The differences between the lines for calmodulin expression were significantly smaller when the induction time was extended, and great variations in induction efficiencies were observed for calmodulin but not for any of the other tested constructs. The reason for the suppression of calmodulin expression is unclear. For chemically competent pACYC-RIL cells, transformation efficiencies of 1 x 10^8 /μg of pUC18 could be achieved, which was the expected efficiency for a BL21goldDE3 derived cell line. Aside from the induction of calmodulin, no negative effects of the pACYC-based tRNA expression arrays on host cell performance have been detected.--

- Replace the paragraph at page 29, lines 10-17 with the following:

--In order to find further examples of genes aided in their bacterial expression by ectopic tRNA expression, six recombinant *Pyrococcus furiosus* genes were tested that are only expressed poorly in *E. coli*. The choice of archaebacterial genes was based on their strong bias for rare *E. coli* codons, especially AGG/AGA and AUA (in fact, 95% of the arginine codons are AGG or AGA). Of six tested constructs, four were rescued by co-expression of the pACYC-RIL construct. The enhanced expression of *Pfu*-polymerase I, *Pfu* polymerase II subunit I, *Pfu* polymerase II subunit II, and *Pfu* pyrophosphatase is shown in Figs. 6 and 9.--

-Replace the paragraph at page 29, line 21 to page 30, line 11 with the following:

--Judging by the codon usage of *Pyrococcus furiosus*, genes from this organism are expected to be affected by AGG/AGA (arginine) and AUA (isoleucine) codons. The sequence of *Pfu* DNA polymerase I contains several pairs of rare arginine and isoleucine codons. In order

to test the effect of the simultaneous presence of extra copies of the *argU and ileY* genes on expression of *Pfu*-polymerase, IPTG-induced expression level of this gene was compared in strains containing extra copies of different combinations of tRNA genes. As shown in Fig. 6, expression of *Pfu* DNA polymerase I is enhanced by the presence of the *argU* gene in the RG set (*argU* and *glyU*) but to a significantly smaller degree than is achieved by a set or array containing both the *argU* and *ileY* genes (RI and RIL). Thus, simultaneous expression of *argU* and *ileY* yielded about 5-fold higher expression of *Pfu* DNA polymerase I than expression on either gene alone. The set containing only the *ileY* gene but not the *argU* gene (IL) failed to enhance expression of *Pfu* polymerase when compared to BL21goldDE3 cells. However, the *argU* gene in the RG set and the *ileY* gene in the IL set were sufficient to rescue expression of constructs only affected by AGG/AGA codons (hcTnT) or AUA codons (CBP-3xi-Cre) to the same level as was observed with the RI and RIL. The lack of efficient *Pfu* DNA polymerase production in host cells containing the RG set was not due to potentially negative effects of the *glyU* tRNA, because the same effect was observed in cells carrying the RL set (Fig. 7).--

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- Replace the paragraph at page 30, lines 12 to 19 with the following:

--As described herein, the expression of the *ileY* gene is compromised in different isolates of the RILP array. Two isolates were used (RILP9, lacking detectable functional *ile Y* and RILP 16, displaying diminished *ile Y* function when compared to RIL) to demonstrate the dose-dependence of *Pfu* DNA polymerase expression on *ileY* expression. As shown in Fig. 8 the expression of *Pfu* DNA polymerase in the RIL and RILP strains correlates with the functional expression of *ileY*. Thus, efficient expression of *Pfu* DNA polymerase in *E.coli* is dependent on the simultaneous presence of extra copies of the *argU* as well as the *ileY* gene.--

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- Replace the paragraph at page 30, lines 20 to 28 with the following

B18

--To test whether the improved expression of the target gene by simultaneous expression of several different tRNA genes is restricted to *Pfu*-polymerase 1, two additional proteins, *Pfu* polymerase II subunit I and subunit II were tested. Judged by their sequence, both genes should be similarly affected. In fact, expression of both genes was enhanced about 5-fold by coexpression of the *argU and glyU* tRNA genes (Fig. 9), similar to the observation with *Pfu* DNA polymerase 1. This suggests that the requirement for extra copies of two or more different